Human Dual IFNγ/IL-17A Fluorospot

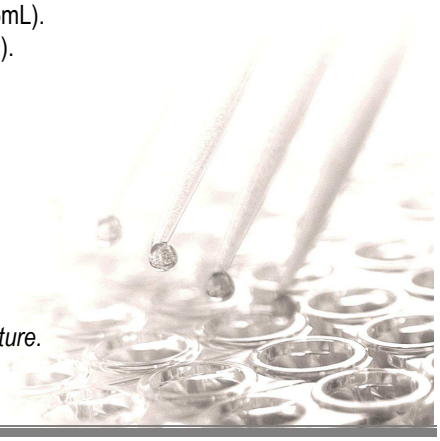
Without plates		With non-sterile plates		With sterile plates	
❒ 874.082.005	5x96 wells	❒ 874.082.001	1x96 wells	❒ 874.082.111	1x96 wells
❒ 874.082.010	10x96 wells	❒ 874.082.005 P	5x96 wells	❒ 874.082.005 S	5x96 wells
❒ 874.082.015	15x96 wells	❒ 874.082.010 P	10x96 wells	❒ 874.082.010 S	10x96 wells
❒ 874.082.020	20x96 wells	❒ 874.082.015 P	15x96 wells	❒ 874.082.015 S	15x96 wells
		❒ 874.082.020 P	20x96 wells	❒ 874.082.020 S	20x96 wells

Intended use

The ELISPOT assay is designed to enumerate cytokine producing cells in a single cell suspension. This method has the advantage of requiring a minimum of *in-vitro* manipulations allowing cytokine production analysis as close as possible to *in-vivo* conditions in a highly specific way. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation, and the follow-up of such frequency during a treatment and/or a pathological state. Elispot assay constitutes an ideal tool in the TH1 / TH2 response, vaccine development, viral infection monitoring and treatment, cancerology, infectious diseases, autoimmune diseases and transplantation.
Diaclone Elispot assay is based on sandwich immuno-enzyme technology. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation or binding on soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates.

Reagents provided (Contents shown for 5x96 wells)

- Capture antibody for IFNγ (0.50 mL). Supplied sterile
- Capture antibody for IL-17A (0.50 mL). Supplied sterile
- FITC conjugated detection antibody for IFNγ (lyophilised, resuspend in 0.55mL).
- Biotinylated detection antibody for IL-17A (lyophilised, resuspend in 0.55mL).
- Anti-FITC antibody green fluorescence conjugate.
- Streptavidin-phycoerythrin conjugate.
- Bovine Serum albumin.
- Fluorenscence buffer (2.5ml).
- 96 PVDF-bottomed-well plates (5 if ordered).



Please note for 1x96 demo kits, detection antibodies are provided in liquid form.
Store all reagents at 4°C
Except plates and Fluorescence buffer which should be stored at room temperature.

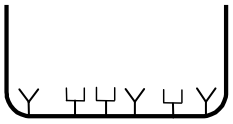
Materials / Reagents not provided

- 96 PVDF-bottomed-well plates. We recommend Millipore MultiScreen plates cat # MSIPN4510 or cat #MSIPS4510
- Cell culture media.
- CO_2 incubator.
- 70% ethanol.
- Tween 20.
- Phosphate buffered saline.
- ELISPOT reading system.

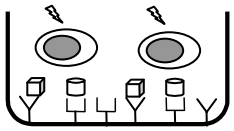
Principle of the method

After cell stimulation, locally produced cytokines are captured by IFNγ and IL-17A specific monoclonal antibodies. After cell lysis, trapped cytokine molecules are revealed by a secondary anti- IFNγ FITC conjugated antibody and a biotinylated anti-IL-17A antibody. Those are in turn recognised by anti-FITC green fluorescent dye and streptavidin-phycoerythrin conjugates. PVDF-bottomed-well plates are then read under a UV light beam. Green fluorescent spots indicate IFNγ production while IL-17A is revealed by red spots. Yellow spots will indicate dual cytokine producing cells.

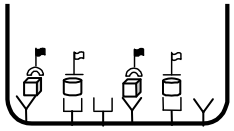
Procedure Summary



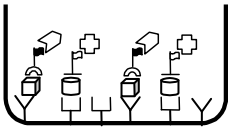
96 PVDF-bottomed-well plates are first treated with 70% ethanol and then coated with anti- IFNγ and anti-IL-17A capture antibodies.



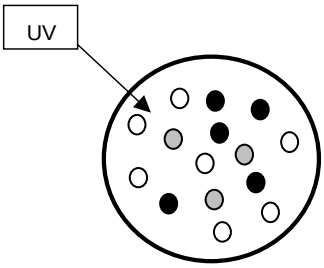
Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines molecules which bind to the capture antibodies.



Cells are lysed. Anti- IFNγ -FITC and anti-IL-17A biotin detection antibodies are added and bind to the captured cytokines.



Detection antibodies are in turn bound by anti-FITC-green fluorescent dye for IFNγ and streptavidin-phycoerythrin for IL-17A.



Finally fluorescent spots are visualised under a UV light beam. Cells producing IFNγ give green spots while those producing IL-17A give red spots. Dual cytokine producing cells give yellow spots.

Assay control

IFNγ/IL-17A production by PBMC upon stimulation by PMA and Ionomycin. This protocol is given as a suggestion.

We recommend to dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated foetal calf serum) containing 1ng/ml PMA and 500ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute from 1.10^5 to $2.5\ 10^4$ cells in antibody coated PVDF-bottomed-wells and incubate for 15-20 hours in an incubator.

However, different conditions may be assayed with varying cells types and stimulating antigens or mitogens. The assay should be optimised in each application.

Reagent preparation

- **Detection antibodies**

Reconstitute each vials with 0.55mL of distilled water. Gently mix the solution and wait until all the lyophilised material is reconstituted.

If not used within a short period of time, reconstitute detection antibody should be aliquoted and stored at -20°C. In these conditions the reagent is stable for at least one year.

Please note for the 1X96 wells, detection antibodies are provided in liquid form.

- **Anti FITC-green fluorescence conjugate / Steptavidin-phycoerythrin :**

Dilute each reagent with the volume indicated on each vial in 10 ml of PBS 1% BSA.
For 1 plate, prepare 10 ml of anti FITC-green fluorescence conjugate / Streptavidin-phycoerythrin solution.

PREPARATION IMMEDIATELY BEFORE USE IS RECOMMENDED.

- **Phosphate buffered saline (10X Concentrate solution).**

For 1 litre weigh: 80g NaCl ; 2g KH_2PO_4 ; 14.4g Na_2HPO_4 $2H_2O$. Add distilled water to 1 litre. Check that pH is comprised between 7.4 +/- 0.1. **This solution should be diluted to 1X before use.**

- **1% BSA in PBS**

For one plate dissolve 0.2 g of BSA in 20 mL of 1X diluted PBS.

- **0.05% Tween in PBS**

For one plate dissolve 35µl of Tween 20 in 70 ml of 1X diluted PBS.

- **35% ethanol in water**

For one plate mix 3.5 ml of ethanol with 6.5 ml of distilled water.

- **5% Fluorescence buffer (optional use)**

For one plate dissolve 500µl of Fluorescence buffer in 10 ml of PBS 1X.

Fluorospot procedure

1. Incubate PVDF-bottomed-well plates with 25µl of 35% ethanol for 30 sec at room temperature.
2. Empty wells and wash three times with 100µ/welll of PBS.
3. Pipette 100µl of IFNγ capture antibody and 100µl of IL-17A capture antibody in 10 mL of PBS. Mix and dispense 100 µl into each well, cover the plate and incubate overnight at +4°C.
4. Empty wells and wash once with 100 µl of PBS.
5. Dispense 100 µl/well of RPMI 10% FCS into wells, cover and incubate for 2 hours at room temperature.
6. Empty wells by flicking the plate over a sink and tapping it on absorbent paper.
7. Wash plate once with PBS.
8. Dispense into wells 100 µl/well of cell suspension containing the appropriate number of cells and appropriate concentration of stimulator. Cells may have been previously *in-vitro* stimulated (Indirect ELISPOT). Cover the plate with a standard 96-well plate plastic lid and incubate cells at 37°C in a CO_2 incubator for an appropriate length of time (15-20 hours). **During this period do not disturb the plate.**
9. Empty wells by flicking the plate over a sink and gently tapping it on absorbent paper.
10. Dispense 100µl of PBS-0.05% Tween 20 into wells and incubate for 10 min at +4°C.
11. Wash wells three times with PBS-0.05% Tween 20.
12. For 1 plate dilute 100µl of reconstituted IFNγ detection antibody and 100µl of reconstituted IL-17A detection antibody into 10 mL of PBS containing 1% BSA. Dispense 100µl into wells, cover the plate and incubate 1 hour 30 min at room temperature.
13. Empty wells and wash three times with PBS-0.05% Tween 20.

14. Distribute 100 µl of anti FITC-green fluorescence conjugate / Streptavidin-phycoerythrin solution (see reagent preparation) in each well. Seal the plate and incubate for 1 hour at room temperature.
15. Empty wells and wash three times with PBS-0.05% Tween 20.
16. Peel off the plate bottom and wash three times both sides of the membrane under running distilled water. Remove all residual buffer by repeated tapping on absorbent paper.
17. Dry wells away from light.
18. Read spots on an Eli-spot reader under a UV light source.
19. Store the plate at +4°C away from light.

Notes

Cell stimulation

Cells can either be stimulated directly in the antibody coated wells (Direct) or, first stimulated in 24 well plates or flask, harvested, and then plated into the coated wells (Indirect).
All the procedure beyond the stimulation step is the same whatever the method (direct/indirect) chosen.

Flurorescence buffer

This buffer will help to increase the fluorescent signal. It can be useful in case of weak fluorescence intensity observed.
Please follow the procedure detailed below:

15. Empty wells and wash three times with PBS-0.05% Tween 20.
16. Distribute 100µl of 5% Fluorescence buffer in each well. Seal the plate and incubate for 15 min at room temperature and away from light.
17. Empty wells by tapping the plate against absorbent paper.
18. Peel off the plate bottom and wash the back of the membrane under running distilled water. **Please note that it is important that the water doesn't get inside the wells**. Remove all residual buffer by repeated tapping on absorbent paper.
19. Dry wells away from light.
20. Read spots on an Eli-spot reader under a UV light source.
21. Store the plate at +4°C away from light.

Anti FITC-green fluorescence / Streptavidin phycoerythrin Conjugates

The quantity of anti FITC-green fluorescence and Strepatvidin-PE conjugates may need adjustements depending on the cell types and on the stimulating antigen studied.

The balance of the 2 different cytokines secreted varies with the cells stimulation. Conjugates dilutions advised in this protocol have been optimised for best results in the suggested protocol (polyclonal activation) .